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PRINCIPAL INVESTIGATOR: Laura Schrader-Kreik, Ph.D.

CONTRACTING ORGANIZATION: Tulane University
New Orleans, LA 70118

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Laura Schrader-Kreik, Ph.D.

E-Mail: schrader@tulane.edu

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14. ABSTRACT
This project investigates the role of gonadal hormones in the regulation of Pavlovian fear conditioning and its extinction. Pavlovian fear conditioning and its extinction serve as an animal model for the development of pathological fear in humans that suffer posttraumatic stress disorders and other anxiety disorders. Despite the increased incidence of PTSD and depressive disorders in women, the specific neurobiological mechanisms of gender differences of PTSD are poorly understood and very little basic research currently investigates this dichotomy. One possible hypothesis is that female hormones also play a role in predisposition to PTSD through epigenetic mechanisms. This concept is currently being tested in this proposal by investigation of the role of gonadal hormones in fear learning and extinction. Contrary to our original hypothesis, we saw no overall effect of gonadal hormones in any of our treatment groups (young female, young male, adult female, adult male). We did, however, observe interesting developmental differences. Young males and adult females exhibited enhanced contextual fear memory relative to young females. Furthermore, young males exhibited enhanced rate of extinction training relative to all groups tested. Investigation of epigenetic regulation in the hippocampus revealed that young males that were extinguished exhibited reduced acetylated H4 relative to young females and adult male and female groups. This is consistent with the behavioral data and provides a target to investigate the role of this modification and genes involved in modulation of extinction rate.

15. SUBJECT TERMS
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INTRODUCTION

This project investigates the role of gonadal hormones in the regulation of Pavlovian fear conditioning and its extinction. Pavlovian fear conditioning and its extinction serve as an animal model for the development of pathological fear in humans that suffer posttraumatic stress disorders and other anxiety disorders. Despite the increased incidence of PTSD and depressive disorders in women, the specific neurobiological mechanisms of gender differences of PTSD are poorly understood, and very little basic research currently investigates this dichotomy. Many theories are proposed to explain the increased incidence of PTSD in females, including an increased predisposition due to prior traumatic experiences. Previous research shows that changes in chromatin structure, or epigenetic mechanisms that regulate transcription of various genes, occur during learning and stress and may contribute to the development of pathological conditions. One possible hypothesis is that female hormones also play a role in predisposition to PTSD through epigenetic mechanisms. This concept is currently being tested in this proposal.

A major problem in PTSD patients is an inability to suppress or inhibit bad memories. Extinction of the conditioned fear response in the laboratory is a simplistic model for debriefing sessions used to reactivate memories of a traumatic experience that have been used as therapeutic strategies for management of PTSD. Investigation of the basic neurobiological mechanisms of the differences in fear acquisition and extinction in males and females and the roles of sex hormones is a strategic target to investigate effective therapies for treatment in both genders. Indeed, behavioral studies in humans have suggested that menstrual cycle and estrogen levels can affect fear acquisition and extinction recall (Milad et al., 2006). These data validate a possible role for hormones in PTSD pathologies.

NOTE – This progress report is an updated report of the report submitted in September. The updated sections are in Task 3 and Task 4 and are denoted by a bullet. The new key research accomplishments are marked by the filled circle rather than the arrow bullet.

BODY

Associative learning in Pavlovian fear conditioning involves training with the presentation of an innocuous stimulus (the conditioned stimulus – CS) that is associated with and predicts an aversive event or the unconditioned stimulus (US). After a brief training period, the subject exhibits a fearful response to the CS. Inhibition or suppression of the fear response and a decline in that conditioned fearful response occur through a complex process called extinction. Extinction of conditioned fear occurs when an organism is repeatedly exposed to the CS in the absence of the US until the conditioned fear response decreases and the fear memory is 'suppressed'.

Research in recent years has greatly increased our understanding of the neural substrates that are involved in fear extinction. Three brain areas and their interconnecting circuitry are highly implicated in the extinction circuitry, including the hippocampus (Bouton et al., 2006), the amygdala (Barad et al., 2006), and the medial prefrontal cortex (Quirk et al., 2006). One area of the medial prefrontal cortex is the infralimbic cortex (IL). The IL contributes the majority of vmPFC inputs to the central nucleus of the amygdala and many of the hypothalamic and midbrain sites that mediate fear responses (Sesack et al., 1989; Hurley et al., 1991). The amygdala, in particular, plays a key role in the expression of conditioned fear (LeDoux, 2003). Therefore, fear conditioning and extinction are useful assays to dissect gender-specific brain area function under various conditions. These behavioral assays are important behavioral tools to determine pathways and molecules or proteins involved in learning and memory.

Fear Conditioning

Fear conditioning involves two components, cued and contextual fear conditioning. Lesion studies showed that the amygdala is involved in both cued and contextual fear conditioning, while the hippocampus also participates in contextual fear conditioning (Kim and Fanselow, 1992; Phillips and

LeDoux, 1992). These studies suggested that the hippocampus plays a role in acquiring and consolidating multimodal representations of contextual fear conditioning. Both spatial learning and contextual learning are thought to be hippocampus-dependent (Kim and Fanselow, 1992; Phillips and LeDoux, 1992).

The role of sex hormones in fear conditioning in rodents has been investigated, with inconsistent results. In male rats, castration reduced memory for contextual but not cued fear conditioning (Edinger et al., 2004); these results suggest an effect of androgens on hippocampus-dependent memory. Other studies, on the other hand, found no effect of testicular hormones on fear conditioning (Anagnostaras et al., 1998).

Various studies have shown a role for ovarian hormones in fear conditioning. OVX female rats display enhanced learning in the contextual fear conditioning paradigm relative to sham-operated as well as estradiol replaced animals (Gupta et al., 2001). This study suggests that estrogen is inhibitory in fear conditioning. Another study suggested that estrogen replacement facilitates both cued and contextual fear conditioning in OVX female mice (Jasnow et al., 2006). Furthermore, estrogen has been shown to enhance other forms of aversive learning, including: trace eyeblink conditioning (Leuner et al., 2004), retention of a passive avoidance task and freezing to a conditioned stimulus (Morgan and Pfaff, 2001, 2002). Various explanations could justify these differences. These include: methodological differences, differences in receptors activated (see below), species differences or developmental differences. In this proposal we study the role of estrogen and development in the fear conditioning and extinction paradigm.

Extinction

The processes that underlie extinction are still controversial. While it is fairly well accepted that extinction is new learning, an erasure of the aversive memory cannot be eliminated (Delamater, 2004; Barad et al., 2006; Lattal et al., 2006; Myers and Davis, 2007); and a combination of the two mechanisms may contribute to extinction. Erasure is a potential mechanism of extinction because recovery of extinguished fear (over time or in a different context) is rarely complete; therefore it appears that a certain amount of 'unlearning' may occur. This 'unlearning' is particularly proposed as a mechanism of extinction in young rats, as young rats do not show renewal or reinstatement of extinguished fear (Kim and Richardson, 2007a, b; Yap and Richardson, 2007). As discussed above, several brain areas, the hippocampus, amygdala and the prefrontal cortex participate in the extinction process. The specific contribution of each to extinction seems to vary depending on species (rat or mouse) and paradigm used.

Very little is known about the role of gonadal hormones in extinction of conditioned fear. The role of testosterone in extinction has not specifically been addressed. The role of estradiol in fear inhibition has been investigated and the effect seems to be dependent on the specific receptor (ER α or β) that is activated. In general it appears that activation of one of these receptors opposes action of the other. One interesting investigation found that estradiol decreased the ability to inhibit fear and discriminate cues in females and this effect is dependent on activation of the ERα receptor (Toufexis et al., 2007). In contrast, adult females in proestrus, which is high levels of estrogen, exhibited an enhanced rate of contextual extinction compared to males and this effect was dependent on activation of the ERβ receptor (Chang et al 2009). Therefore the role of estrogen in extinction may be dependent on differential receptor activation. Recent evidence suggests that that ERα activates and Erβ inhibits similar gene targets and this regulation may be mediated by epigenetic regulation.

We investigated the role of gonadal hormones, estrogen and progesterone, in females and androgens in males, in fear conditioning and its extinction. We hypothesized that estrogen may predispose females to enhanced fear conditioning or a reduced rate of extinction. Conversely, testosterone in males could reduce fear conditioning and enhance extinction rate. We also investigated whether some of these effects may be through epigenetic regulation. In this study, our goal was to define a role for gonadal hormone in fear conditioning or extinction. While small effects were seen in some cases, we saw no significant effects of gonadal hormones. Interestingly, we did observe developmental differences and these effects are different between males and females.

STATEMENT OF WORK

We have conducted experiments that address each of the tasks outlined in the statement of work. The results from each of the experiments are discussed in each task section.

Task 1 –Hypothesis: Sex hormones play a role in extinction (months 1-6)

Methods

Subjects were male and female 129svE mice from Charles Rivers ranging in age from 4 to 14 weeks and all experiments were performed within the guidelines of the Tulane University Institutional Animal Care and Use Committee and ACURO. Mice were housed in groups of three or four on a 12 h light-dark cycle with access to food and water ad libitum.

Gonadectomy

Mice were anesthetized by intraperitoneal injection of a ketamine (80 mg/kg) and xylazine (8 mg/kg) mixture. Gonadectomies were performed at either 4 weeks (young) or 10 weeks of age (adult).

Males: Orchidectomy was performed through a single transverse incision across the end of the scrotum. The testicles were removed and a ligature was used around the vas deferens and spermatic vessels to prevent hemorrhage. Sham animals received similar incisions with no testicle removal.

Females: Ovariectomies were performed via small bilateral dorsal flank incisions and subsequent removal of ovaries. Sham animals received similar incisions with no ovary removal.

Estradiol replacement

One group of ovariectomized mice received 200 nM 17β–estradiol in their drinking water. This concentration was shown to be a safe and effective way to produce uterotrophic responses in ovariectomized mice (Levin-Allerhand et al, 2003).

Behavior

Fear Conditioning

Fear conditioning was performed using a computer-controlled, sound-attenuated, conditioning chamber (29x19x25 cm). On the day of the fear conditioning training the mouse was exposed to the chamber for the first time and allowed to explore for 150 s. At 150 s the mouse received one 2 s electrical foots hock (0.5mA) through a stainless steel grid floor. The foot shock was preceded by a 30 s tone. After the foot shock the mouse was allowed to explore the context for an additional 150 s. The behavior of the mouse was measured by a digital infrared video camera mounted in front of the chamber.

Context-dependent freezing

24 hours after fear conditioning the mouse was returned to the chamber to measure context-dependent fear conditioning. The mouse was placed in the chamber for 300 s and the degree of freezing was analyzed using Video Freeze Software.

Cued freezing

48 hours after initial fear conditioning mice were returned to the chamber with an altered context. After 150s they were exposed to the tone again and the degree of freezing was measured.

Shock Threshold and Fear Extinction
Mice were placed in the fear conditioning chamber and allowed to explore for 30 s before being exposed to a series of foot shocks. The foot shocks lasted 2s and ranged in intensity from 0.1 to 0.7 mA. The behavior of the animals was recorded and responses were scored in order to determine differences in shock sensitivities. 48 hours after shock threshold, mice were returned to the chamber for 5 min to monitor freezing. This was repeated for 4 days and the degree of freezing was plotted to monitor extinction of fear.

Progress to date:
Task 1 – To study the role of hormones in extinction using fear conditioning and extinction protocols. We have completed these experiments. We have conducted this experiment on male and female mice that were gonadectomized (GDX) in adulthood and animals that were GDX before puberty. The different ages were addressed previously in the statement of work as a possible alternative to study the developmental and programmatic roles of gonadal hormones. In addition to fear conditioning and extinction, we studied the role of hormones in regulation of various anxiety behaviors and foot shock response as a control for the fear conditioning and extinction as increased anxiety or footshock sensitivity could confound these results. The results from each test are presented in Table 1 for males and Table 2 for females. Figures are in the appendix and are referenced in the statistics section. We saw very few significant results in response to GDX in either sex. The largest effects that we observed were sex and developmental differences in contextual fear conditioning and extinction.

Adult males –
Castration causes shrinkage of the bulbospongiosus muscles (BSM) (Wainman and Shipounoff, 1941). Castrations were confirmed upon sacrifice by comparison of BSM weight in sham and castrated animals. BSM weight in castrated males (n = 7; 42.5 ± 1.4 mg) was significantly decreased compared to sham males (n = 9, 58.3 ± 2.6 mg; p = 0.0002, unpaired t-test). One castrated male was discarded from the study due to ineffective castration. Anxiety behavior was assayed using two commonly used tests of anxiety: open field assay (OFA) (Weiss et al., 2000) and elevated plus maze (EPM) (Itoh et al., 1991; Kulkarni and Sharma, 1991). These tests are based on a rodents' proclivity to avoid well-lit, open spaces. More anxious animals will spend less time in the center of the open field and in the open arms of the elevated plus maze. Castration caused a significant increase in anxiety in the adult males on the EPM, suggesting castration increased anxiety in adult males. In addition we saw no significant effect of castration on the fear conditioning or extinction paradigms (see Table 1 and statistics following table 1). Figures for each of the tests can be found in the appendix.

Table 1

Males	EPM (% time spent in open arms)	OFA (% time spent in center)	CXT (% time spent freezing)	CUED (% time spent freezing)
Young sham	n = 10; 16 ± 6.5	n = 10; 3.2 ± 2	n =10; 32.3 ± 5.1	n = 10; 59.3 ± 4.4
Young castrated	n = 10; 32 ± 7.6	n = 10;11.9 ± 3.6 *	n = 9; 25.4 ± 1.2	n = 10; 43.9 ± 9.1
Adult sham	n = 9; 29.8 ± 5.9	n = 9; 7.7 ± 4.6	n = 9; 37.4 ± 5.9	n = 9; 56.8 ± 6.5
Adult castrated	n = 7; 8.0 ± 3.5*	n = 7; 12.0 ± 5.1	n = 7; 30.5 ± 5.0	n = 7; 53.1 ± 7.2

Statistics for Adult Males:
Anxiety
(EPM): *, unpaired t-test, p = 0.011 (Figure 1A).
Open Field Assay (OFA) unpaired t-test, p = 0.66 (Figure 1B).
Total distance traveled (meters): sham n = 9, 36.5 ± 10.2; castrated n = 7, 32.7 ± 9.2; p = 0.79

Shock threshold – The response to an increasing intensity of foot shock was measured (Alexander et al., 2009) and compared between groups. Two Way Anova revealed no significant effect of treatment (castration): $F_{(1,112)} = 0.59$, $p = 0.46$, however, a significant effect of intensity was observed as expected $F_{(8,112)} = 109.2$, $p < 0.0001$ (Figure 1C).

Fear conditioning – amount of freezing during test period (24 hours later for context, 48 hours for cued)
Cued – unpaired t-test; $p = 0.72$ (Figure 2A)
Context – unpaired t test $p = 0.41$ (Figure 2B)
Extinction - (see figure 2C) - Two Way Anova – There was no significant effect of treatment (surgery) - ($F_{(1,42)} = 0.38$, $p = 0.55$); however, as expected there was a significant effect of day ($F_{(3,42)} = 25.5$, $p < 0.0001$).

Young males
Castration was confirmed in the young males using BSM mass as described above. Castration caused a significant decrease in BSM mass (n = 10; castrated - 27.8 ± 3.0, $p = 0.03$) compared to sham-operated (n=10; 37.9 ± 3.1 mg). Anxiety tests revealed significantly less anxiety on OFA and a trend toward less anxiety in the castrated young males. These data combined with the anxiety data from the adult males suggest that GDX has different effects on anxiety in the young and adult males. No other significant effects of castration were observed in the young males.

Anxiety
EPM $p = 0.14$ (Figure 3A)
OFA *, $p = 0.05$ (Figure 3B)
Total distance traveled (meters): sham n = 10, 13.3 ± 3.4; n = 10, castrated 16.5 ± 4.3; $p = 0.57$
Shock threshold – No significant effect of treatment: $F_{(1,144)} = 2.71$, $p = 0.12$; Significant effect of stim intensity – $F_{(8,144)} = 227.8$; $p < 0.0001$ (Figure 3C).

Fear Conditioning
Context – unpaired t test – $p = 0.23$ (Figure 4A)
Cued – unpaired t-test – $p = 0.14$ (Figure 4B)
Extinction - No significant effect of treatment: $F_{(1,48)} = 1.8$, $p = 0.20$; Significant effect of day $F_{(3, 48)} = 81.31$ $p < 0.0001$ (Figure 4C).

Table 2

Females	**EPM (% time spent in open arms)**	**OFA (% time spent in center)**	**CXT (% time spent freezing)**	**CUED (% time spent freezing)**
Young sham	n = 10; 27.8± 6.5	n = 10; 4.5 ±1.6	n = 10; 22.9 ± 4.1	n = 10; 50.0 ±6.1
Young OVX	n = 10; 43.0±9.9	n = 10; 7.9±3.2	n = 10; 19.2 ± 3.6	n = 10; 41.6 ±7.6
Young OVX+E	n = 10; 18.2 ± 7.0	n = 10; 7.1± 2.6	n = 10; 28.6 ± 6.0	n = 10; 45.4 ± 8.4
Adult sham	n = 6; 26.3 ± 5.7	n = 6; 13.3 ± 3.7	n = 6; 36.2 ± 10.4	n = 6; 41.2 ± 11
Adult OVX	n = 6; 20.7 ± 9.3	n = 6; 7.2 ± 2.6	n = 6; 38.1 ± 5	n = 6; 54 ± 5
Adult OVX+E	n = 5; 12.6 ± 6.3	n = 5; 8.5 ± 2.0	n = 5; 43 ± 10	n = 5; 49 ± 8

Adult females
OVX causes a significant reduction of uterine weight; therefore, successful OVX was confirmed with a decrease in uterine weight. OVX - n=6, 5 ± 0.2 mg; sham n=6, 40 ± 8 mg; OVX+E n = 5, 11 ± 0.6 mg.
No significant effects of gonadectomy or estradiol replacement were observed in any of the behavioral measures, although a trend toward an effect on shock threshold was observed. This did not appear to

affect fear conditioning or extinction. The method of estradiol administration may have been an issue. Attempts to measure circulating estradiol levels were unsuccessful. Our replacement method had a relatively small effect on uterine weight compared to OVX. Future studies should use a different method of estradiol administration.

Anxiety
EPM - $F_{(2,14)} = 0.82$, $p = 0.46$ (Figure 5A)
OFA - $F_{(2,14)} = 1.27$, $p = 0.31$ (Figure 5B)
Total distance traveled (meters) - Sham – n = 6, 47.8 ± 5.8; OVX n = 6, 28.0 ± 14; OVX+E n = 5, 12.9 ± 1.9; $F_{(2,14)} = 3.35$; $p = 0.07$
Shock threshold – No significant effect of treatment- $F_{(2,112)} = 2.94$, $p = 0.09$; significant effect of stim intensity $F_{(8, 112)} = 153.7$; $p < 0.0001$ (Figure 5C).

Fear Conditioning –
Context $F_{(2,14)} = 0.16$, $p = 0.86$ (Figure 6A)
Cued – $F_{(2,14)} = 0.57$, $p = 0.58$ (Figure 6B)
Extinction – treatment $F_{(2, 42)} = 0.28$, $p = 0.76$; time $F_{(3, 42)} = 28.5$; $p < 0.0001$ (Figure 6C)

Young females
No significant effect of gonadectomy or estradiol replacement was observed in any of the measures, although a trend toward an effect on shock threshold was observed. This did not appear to affect fear conditioning or extinction as no significant effects were observed.
Successful OVX and E replacement was confirmed with uterine weight: OVX, n = 10, 3.8 ±0.3mg; OVX+E, 23.4±7.7 mg; sham 54.3± 3.3 mg.

Anxiety –
EPM - $F_{(2,27)}$; = 2.5 $p = 0.1$ (Figure 7A).
OFA - $F_{(2,27)} = 0.4818$ $p = 0.62$ (Figure 7B).
Total distance traveled (meters) - Sham – n = 10: 27.3 ± 6.6; OVX n = 10; 37.9 ± 9.9; OVX+E n = 10, 43.2 ± 12.5 $F_{(2,27)} = 0.66$; $p = 0.52$
Shock threshold - No effect of treatment $F_{(2,216)} = 2.906$, $p = 0.07$; Significant effect of stim intensity; $F_{(8,216)}$ 308.5, $p < 0.0001$ (Figure 7C).

Fear Conditioning -
Context –$F_{(2,27)} = 1.0$; $p = 0.37$
Cued - $F_{(2,27)} = 0.32$; $p = 0.73$
Extinction - No significant effect of treatment - $F_{(2,81)} = 0.031$ $p = 0.97$; Significant effect of stimulus intensity $F_{(3, 81)} = 66.75$; $p < 0.0001$.

Overall summary
We saw no effect on GDX or estrogen in females on extinction or cued or contextual fear conditioning in any group tested. There was a significant effect of GDX on the measures of anxiety in the young male with castration causing a significant decrease in anxiety. Several interesting sex and developmental differences have arisen, however. Since GDX had no significant effect in the young animals, we included both sham and GDX in our analysis. We found that young males exhibited significantly enhanced contextual fear conditioning (n = 19; 29 ± 2.8) relative to young females (n = 20; 21 ± 2.7; $p = 0.045$, t-test, figure 9A) regardless of surgery condition (GDX or sham). This suggests that young males have enhanced contextual fear memory relative to females. Furthermore, young females exhibited significantly less contextual fear memory (n=20; 21 ± 2.7) compared to adult females (n = 12; 37.2 ± 5.5; $p = 0.006$,

figure 9B). Interestingly, when all groups (young male, adult male, young female and adult female) were compared on extinction training rate, a significant difference emerged, with young males extinguishing faster than other groups ($F_{(3,312)} = 23.68$; $p < 0.001$; figure 9C).

Task 2 – Blockade of histone deacetylases can modulate extinction (months 6-12)

This experiment is similar to task 1 and we will test the role of histone deacetylases in extinction. In this task, we will determine whether sodium butyrate, a histone deacetylase inhibitor, can modulate extinction in intact and gonadectomized animals. The effect of inhibition of histone deacetylases during training on extinction will be determined. We hypothesized that hormones regulate histone acetylation (tested in task 3 and 4) to modulate extinction, however, given the data described above, the most parsimonious hypothesis is that histone acetylation is developmentally regulated.

- We tested systemic injection of sodium butyrate (SB, 0.8 mg/kg), a histone deacetylase inhibitor one hour before the initial extinction training trial (Figure 12A). While the effect of SB was not significant, we did see a trend toward an enhanced rate of extinction training $F_{(1,18)} = 2.53$, $p = 0.13$. We hypothesized that repeated injection of SB before re-exposure may be necessary to enhance extinction. Therefore we injected SB 1 hour before the extinction on day one and also on the re-exposures on days 2-4. Injection of SB (0.8 mg/kg) one hour before the re-exposures was not significantly different than saline-injected (Figure 12B) ($F_{(1,8)} = 0.001$; $p = 0.97$). We also attempted a SB injection 3 hours before extinction, similar results were obtained, with SB-injected not significantly different from saline-injected (Figure 12C, $F_{(1,8)} = 0.04$; $p = 0.86$). We concluded that SB had no effect on extinction.

- In a closer observation, the saline-injected animals extinguished faster than the control adult male mice previously observed (figure 2C). The effect of the 3 hour injection on day 1 freezing was particularly striking. Therefore, we hypothesized that the injection caused an acute stress response that enhanced fear memory suppression. We further compared freezing on day 1 of extinction in the animals that were injected with saline 3 hours, 1 hour and non-injected animals (Figure 12D). Interestingly, animals injected 3 hours prior to the first re-exposure froze significantly less than either the animals injected 1 hour prior or the non-injected group $F_{(2,26)} = 6.725$; $p = 0.004$. This suggests a time-sensitive period when an acute stress can enhance fear memory suppression. While we did not investigate the mechanisms of this effect, an acute increase in corticosterone caused by the injection may explain this effect. This suggests that corticosterone exposure before re-exposures may enhance fear memory suppression. Further studies will investigate this effect.

Task 3 – Hormones mediate changes in chromatin structure and alter epigenetic effects that occur during acquisition of fear memories.

We studied acetylation of histone H3 and H4 at two sites that we have previously been reported to be involved in memory (Levenson et al., 2004) and that we observed were modulated by stress. Preliminary data suggest that there is no effect across the male and female treatment groups in adult animals (figure 10 – females; figure 11 –males). This is consistent with our observations described above that hormones do not affect fear conditioning or extinction.

Females - There was no significant difference ($F_{(2,17)} = 0.22$; $p = 0.8$) between sham (n = 7; 100 ± 35%), OVX (n = 7; 75 ± 26), and OVX+E (n = 6; 75 ± 30%) for pAcH3. In addition, there was no significant effect for acetylated lysine 12 on H4 ($F_{(2,17)} = 1.4$; $p = 0.27$); sham (n =7; 100 ± 8.7%), OVX (n = 6; 100.8 ± 15.5%), OVX+E (n = 7; 124.7 ± 11.7%).

Males - pAcH3 - sham (n = 10; 100 ± 24%), castrated (n = 8; 111.4±32.5%) p = 0.78

Acetyl H4 – sham (n = 10; 100 ± 9.5%), castrated (n = 9; 104.3 ±14.2%) p = 0.8

Therefore, it does not appear that hormones affect histone acetylation in the hippocampus.

Task 4 – Epigenetic changes during acquisition of the fear response are modulated by hormones (months 12-18).

- These experiments are currently ongoing and a no cost extension has been requested to complete these experiments. We compared acetylation of H3 and H4 in the hippocampus of young females, young males, adult females and adult males that had been exposed to extinction (Figure 13). We found that acetylation of H3 is not significantly different in any of our groups (Figure 13A; $F_{(3,11)}$ = 1.05; p = 0.42). Interestingly, however, acetylated H4 is greatly reduced in young males, although the difference is not yet significant (Figure 13B; F(3,14) = 2.78; p = 0.08). This correlates with a rapid extinction in the young males. Acetylated H4 provides an epigenetic modification target to investigate using the chromatin immunoprecipitation assay to investigate genes that are regulated by this modification.

KEY RESEARCH ACCOMPLISHMENTS

- Castration decreases anxiety in young males and increases anxiety in adult males.
- Male adolescent mice exhibit enhanced memory for contextual fear conditioning compared to female adolescent mice.
- Adult female mice exhibit greater memory for contextual fear conditioning than adolescent female mice.
- Male adolescent mice extinguish fear memory more rapidly than adolescent female, adult male and adult female mice.
- No differences in histone modifications between adult treatment groups. This result is consistent with our lack of effect in behavior.
- Administration of the histone deacetylase inhibitor (sodium butyrate 0.8 mg/kg) had no effect on extinction.
- Acetylation of histone 4 was decreased in the young males. This modification correlated to a rapid extinction and provides a possible molecular substrate for further investigation into genetic regulation associated with fear memories and extinction.

REPORTABLE OUTCOMES

MHRF meeting poster and presentation in symposium:

Hormonal Regulation of Extinction: Implications for the Mechanisms of Gender Differences in PTSD, Carmel M McDermott and Laura A. Schrader

Manuscripts –

Developmental regulation of extinction: gender differences. In preparation for submission to Neuroscience.

Lack of effect of gonadal hormones on fear conditioning and extinction in male and female mice. In preparation for Hormones and Behavior.

Grant applications

R21 application to be submitted 6/15/10 – Developmental regulation of sex differences in extinction.

CONCLUSION

In conclusion, contrary to our original hypothesis, we saw no overall effect of gonadal hormones in any of our treatment groups (young female, young male, adult female, adult male); although future studies should utilize an increased dose of estradiol replacement in the females. We did, however, observe interesting developmental differences. The young males extinguished fear more rapidly than the other groups. We found that administration of the histone deacetylase inhibitor in adult males had no effect on extinction (Figure 13). Interestingly, injections appeared to provide a stressful stimulus and aid in fear memory suppression. We found that acetylation of H4 was greatly reduced in young males, and this correlated to the group that exhibited enhanced extinction. These studies should establish a genetic marker to investigate as a mechanism for the observed enhanced rate of extinction training in young males.

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Figure 1. Anxiety, foot shock sensitivity and training results in adult male mice. A. Bar graph showing the results of the time spent in the open arms of the EPM in both sham and castrated adult males. Castrated mice spent significantly less time in the open arms (*, $p < 0.05$). B. Bar graph showing the results from the OFA. Percentage of time spent in the center of the field is shown. C. The average response of sham and castrated mice to increasing intensity of stimuli. Responses were graded on a scale of 1-5 as previously described (Alexander et al 2009). D. Bar graph showing the percentage of time spent freezing on training day before the foot shock (left) and after the foot shock (right). Mice displayed normal exploratory behavior, characterized by very little freezing before the foot shock. Foot shock increased the amount of freezing during the last 2 minutes of training in both groups, however castrated mice showed significantly less freezing relative to sham-operated (*, $p < 0.05$).

Figure 2. Contextual and cued fear conditioning and extinction training results from adult male mice. A. Bar graph showing the amount of time spent freezing in response to the context 24 hours after training. B. Bar graph showing the amount of time spent freezing in response to the cue (white noise) 48 hours after the training (left) and freezing in the novel context (right). C. Results from extinction training. Animals were tested for foot shock sensitivity and then 48 hours later received extinction training, consisting of context exposure with no US, for 5 minutes a day over a 4 day period. No significant differences between sham and castrated animals were seen in any of the above results.

Figure 3. Anxiety, foot shock and training results in young male mice. A. Results of time spent in the open arms of the EPM in both sham and castrated young males. B. Results from OFA. Percentage of time spent in the center of the open field is shown. Castrated males spent significantly more time in the center (*, $p < 0.05$), suggesting decreased anxiety in the young castrated mice. C. The average response of sham and castrated mice to increasing intensity of stimuli. Responses to each foot shock were graded on a scale of 1-5. D. Bar graph showing the percentage of time spent freezing on training day before the foot shock (left) and after the foot shock (right). Mice displayed normal exploratory behavior before the foot shock. Foot shock increased the amount of freezing during the last 2 minutes of training in both groups, however castrated mice showed significantly less freezing relative to sham-operated ($p < 0.05$).

Figure 4. Contextual and cued fear conditioning and extinction results from young male mice. A. Bar graph showing the amount of time spent freezing in response to the context 24 hours after training. B. Bar graph showing the amount of time spent freezing in response to the cue (white noise) 48 hours after the training (left) and freezing in the novel context (right). C. Results from extinction training in young male mice. Animals were tested for foot shock sensitivity and then 48 hours later received extinction training for 5 minutes a day over a 4 day period. No significant differences between sham and castrated animals were seen in any of the above results.

Figure 5. Anxiety, foot shock and training results from adult female mice. A. Results of time spent in the open arms of the EPM in sham, OVX and OVX+E adult females. B. Results from OFA. Percentage of time spent in the center of the field is shown. C. The average response of sham, OVX and OVX+E mice to increasing intensity of stimuli. A trend toward increased sensitivity in the OVX females was observed. D. Bar graph showing the percentage of time spent freezing on training day before the foot shock (left) and after the foot shock (right). Female mice displayed normal exploratory behavior before the foot shock. Foot shock increased the amount of freezing during the last 2 minutes of training.

Figure 6. Contextual and cued fear conditioning and extinction results from adult female mice. A. Bar graph showing the amount of time spent freezing in sham, OVX and OVX+E in response to the context 24 hours after training. B. Bar graph showing the amount of time spent freezing in response to the cue (white noise) 48 hours after the training (left) and freezing in the novel context (right). C. Results

from extinction training. Animals were tested for foot shock sensitivity and then received extinction training for 5 minutes a day over a 4 day period. No significant differences between sham, OVX and OVX+E were seen in any of the above results.

Figure 7. Anxiety, foot shock and training results from young female mice. A. Results of time spent in the open arms of the EPM in sham, OVX and OVX+E adult females. B. Results from OFA. Percentage of time spent in the center of the field is shown. C. The average response of sham, OVX and OVX+E mice to increasing intensity of stimuli. D. Bar graph showing the percentage of time spent freezing on training day before the footshock (left) and after the footshock (right). Female mice displayed normal exploratory behavior before the footshock. Footshock increased the amount of freezing during the last 2 minutes of training.

Figure 8. Contextual and cued fear conditioning and extinction results from young female mice. A. Bar graph showing the amount of time spent freezing in response to the context 24 hours after training. B. Bar graph showing the amount of time spent freezing in response to the cue (white noise) 48 hours after the training (left) and freezing in the novel context (right). C. Results from extinction training. Animals were tested for foot shock sensitivity and then received extinction training for 5 minutes a day over a 4 day period. No significant differences between sham, OVX and OVX+E were seen in any of the above results.

Figure 9. Summary of observed developmental differences. A. Young female mice (sham and OVX combined) showed significantly less freezing in the training context compared to adult female mice (**, $p < 0.01$). B. Young female mice (sham and OVX) spent significantly less time freezing in the training context compared to young male mice (sham and castrated). C. Extinction training results from each of the age groups. Young male mice displayed enhanced rate of extinction during the training period compared to the other groups.

Figure 10. Gonadectomy or estrogen replacement had no significant effect on acetylation of lysine 12 on H4 or phospho-acetylation of ser9 lys10 on H3 in adult females. Example western blot probed with phospho-acetyl H3 (A, above) or total H3 antibodies (A, below) or acetylated lys 12 on H4 (B, above) or total H4 antibodies (below). C. Summary bar graph showing changes in phospho-acetylation of H3 (left) or acetylation of H4 (right) relative to sham controls.

Figure 11. Castration had no significant effect on acetylation of lysine 12 on H4 or phospho-acetylation of ser9 lys10 on H3 in adult males. Example western blot probed with phospho-acetyl H3 (A, above) or total H3 (A, below) or acetylated lys12 on H4 (B, above) or total H4 (below). C. Summary bar graph showing changes in phospho-acetylation of H3 or acetylation of H4 relative to sham controls.

Figure 12. Injection of the histone deacetylase inhibitor, sodium butyrate (SB, 0.8 mg/kg) had no significant effect on extinction. A. Injection of SB 1 hour before re-exposure on day 1 had no significant effect on extinction rate, although a trend toward an enhanced extinction was observed. B. Injection of SB one hour prior to exposure every day had no significant effect on extinction rate relative to saline-injected. C. Injection of SB 3 hours prior to extinction had no significant effect on extinction relative to saline-injected. D. Animals injected with saline 3 hours prior to exposure on day 1 froze significantly less than animals injected 1 hour or not injected before re-exposure.

Figure 13. Acetylation of H4, but not H3, is significantly reduced in young males. Acetylation of H3 and H4 was investigated in extinction-exposed animals. A. Acetylation of H3 relative to total H3 was not significantly different across groups. B. Acetylation of H4 relative to total H3 was not significantly

different across groups, however, there was a trend toward reduced acetylation of H4 in the rapidly-extinguishing young males.

ADULT MALES

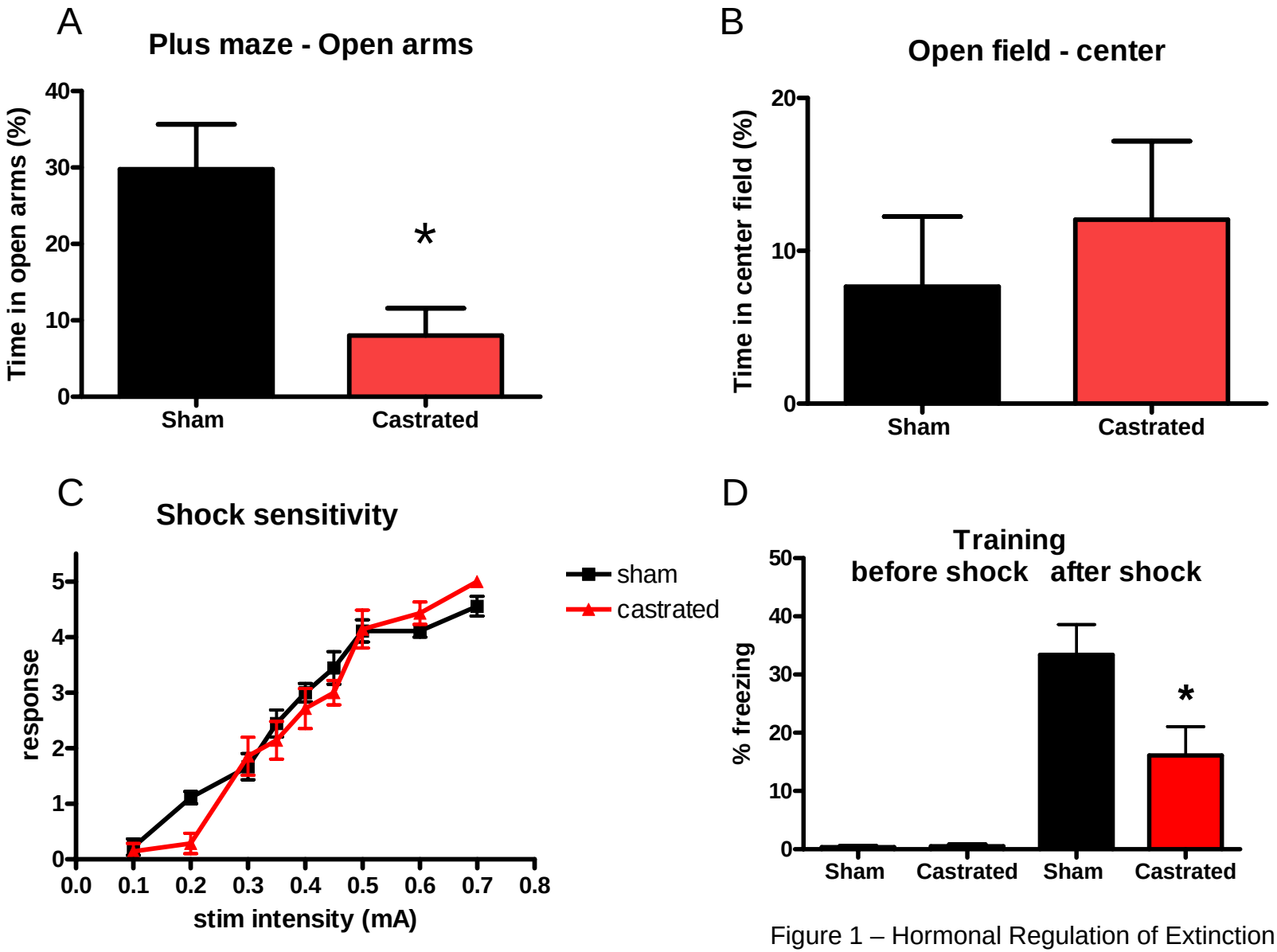


Figure 1 – Hormonal Regulation of Extinction

ADULT MALES

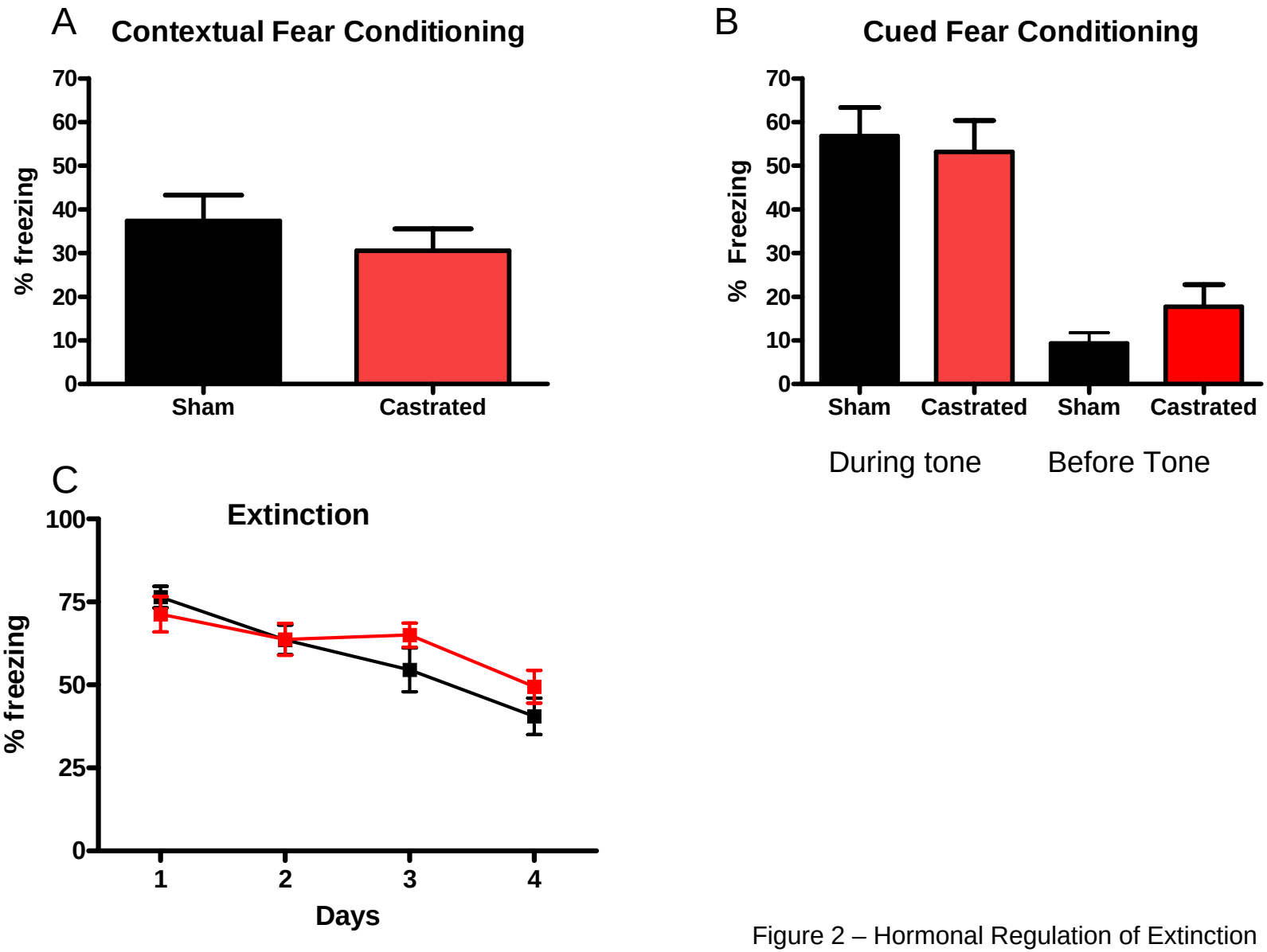


Figure 2 – Hormonal Regulation of Extinction

YOUNG MALES

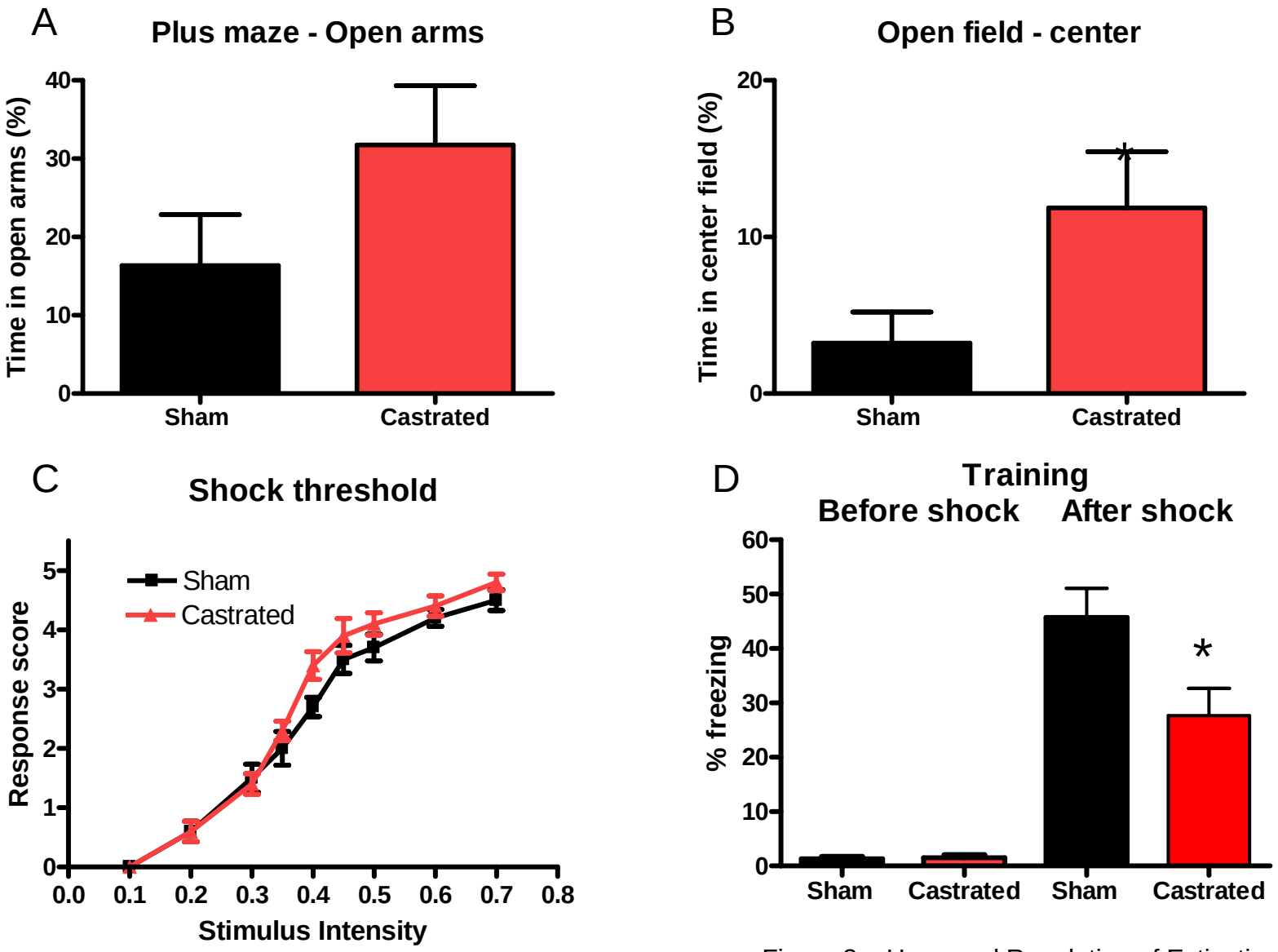


Figure 3 – Hormonal Regulation of Extinction

YOUNG MALES

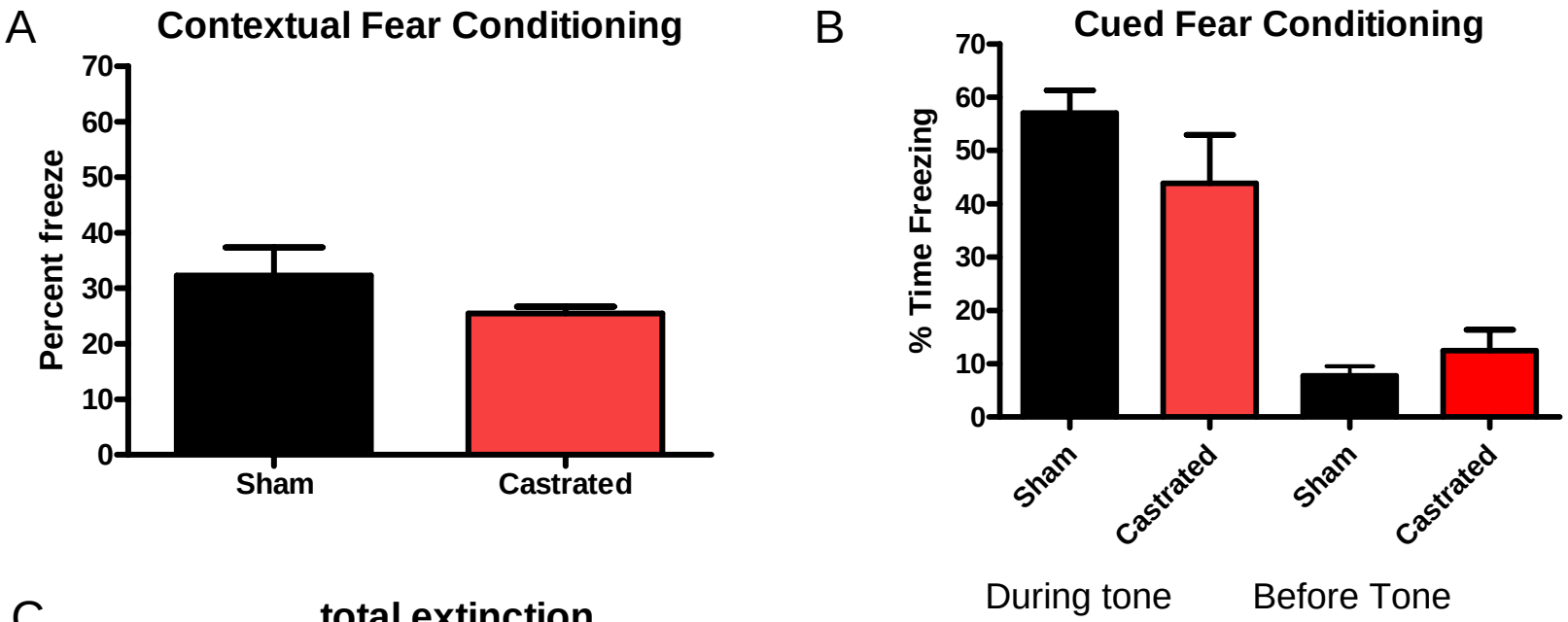


Figure 4 – Hormonal Regulation of Extinction

ADULT FEMALES

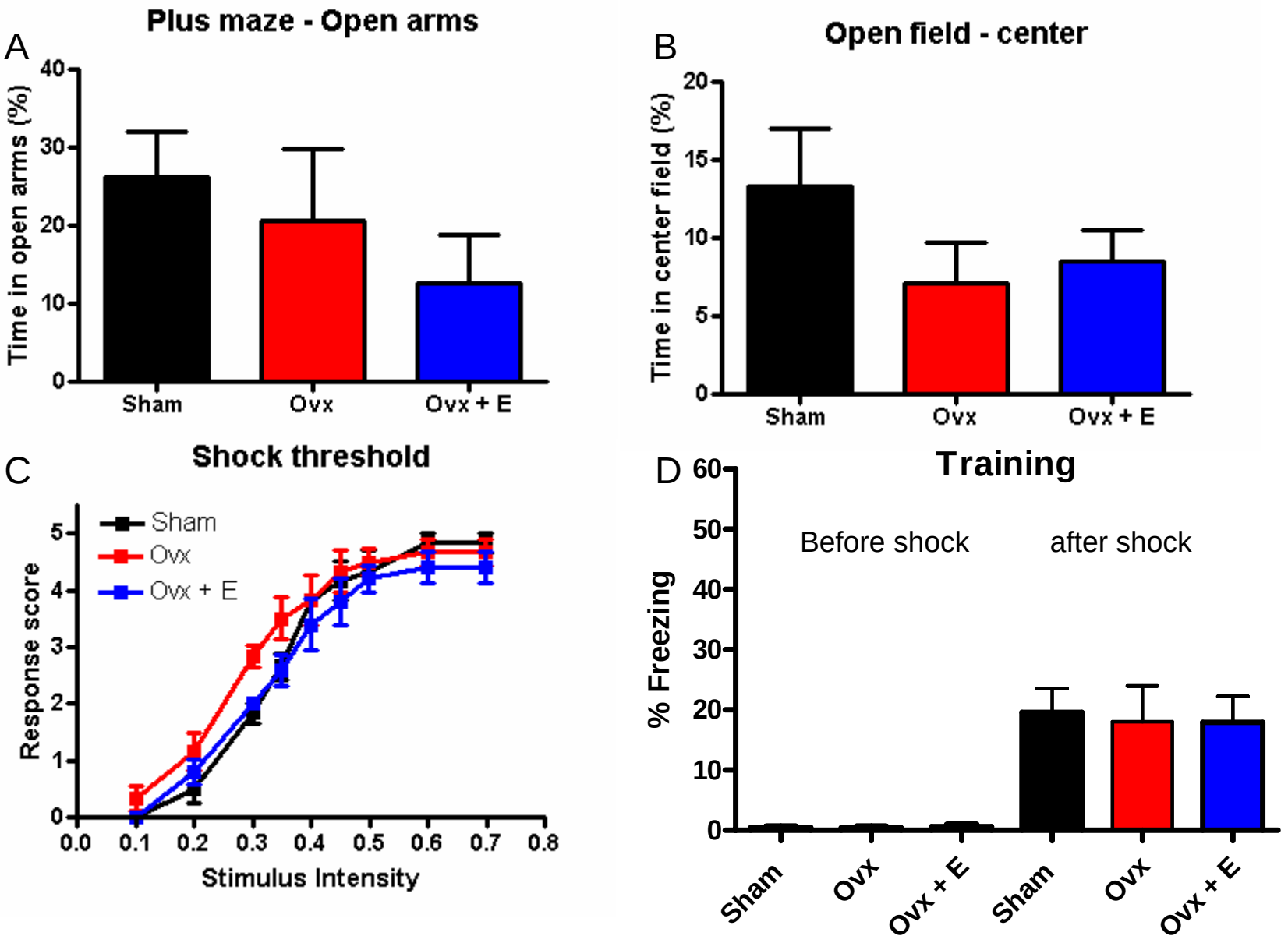
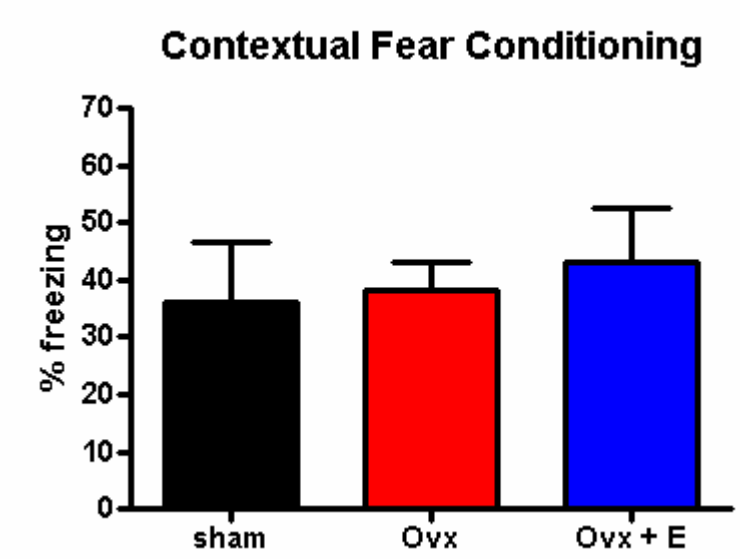


Figure 5– Hormonal Regulation of Extinction

ADULT FEMALES

A



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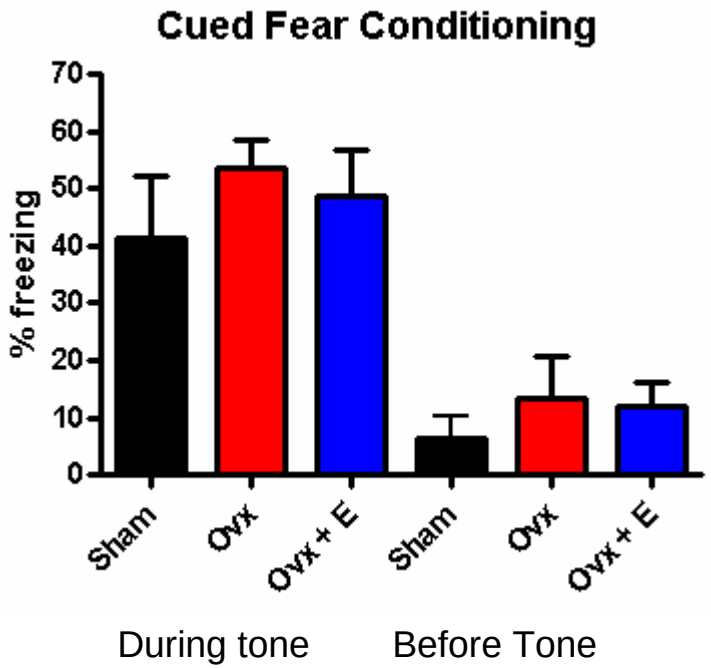

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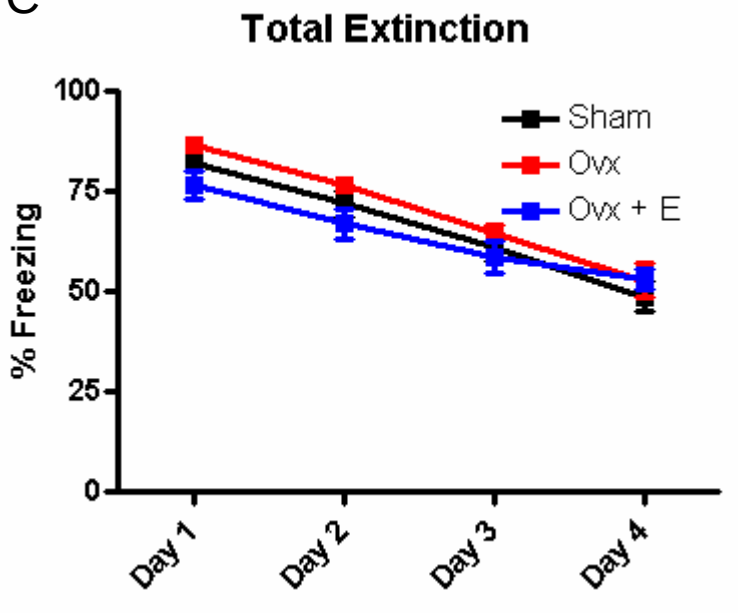


Figure 6 – Hormonal Regulation of Extinction

YOUNG FEMALES

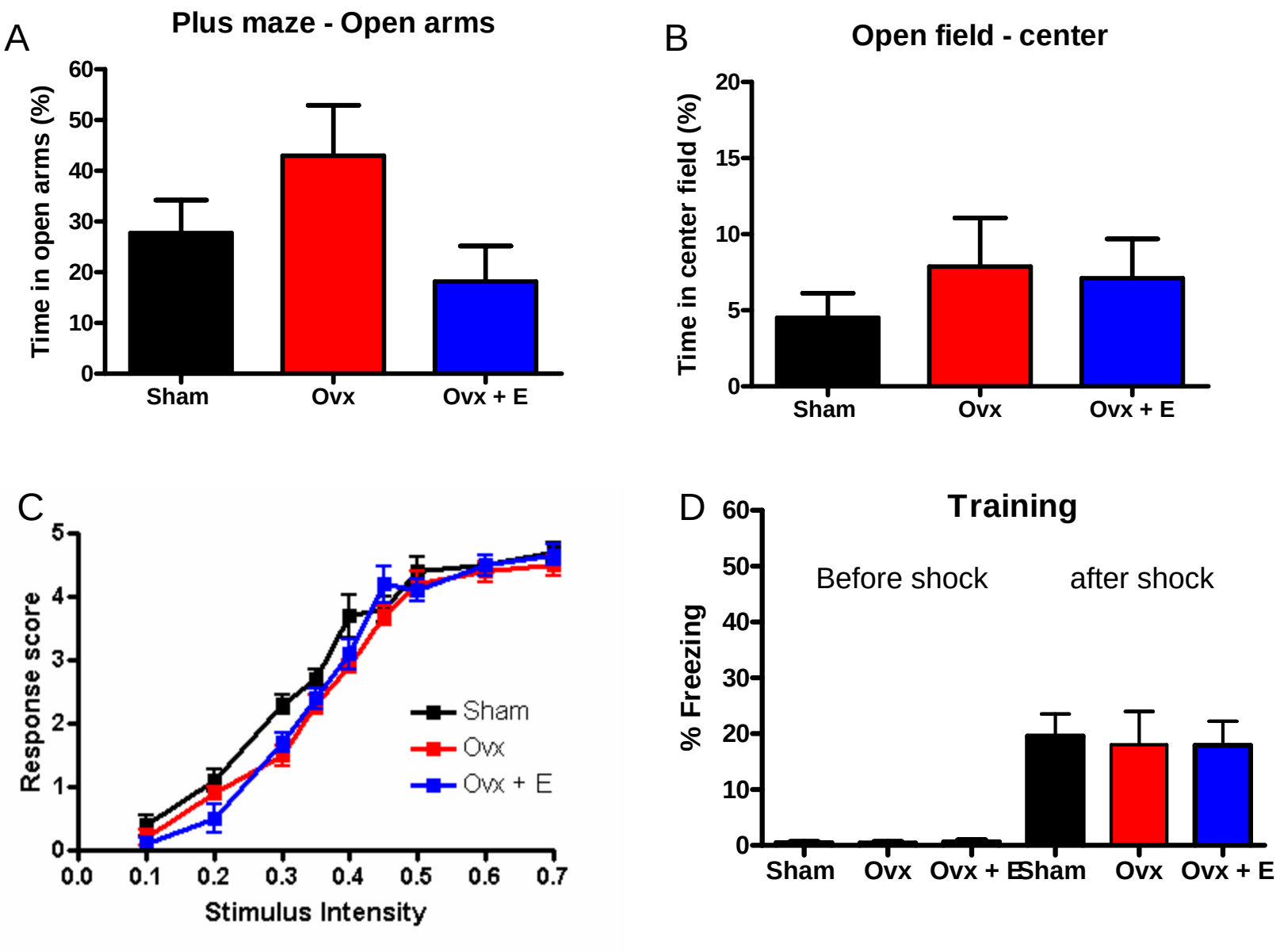
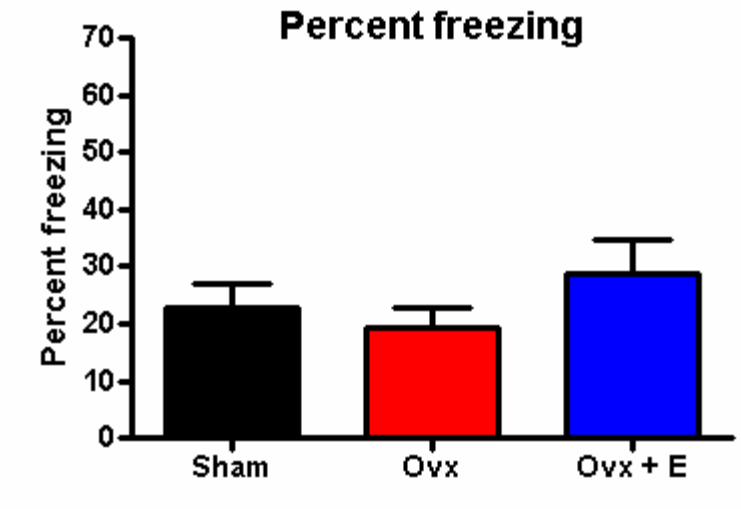


Figure 7 – Hormonal Regulation of Extinction

YOUNG FEMALES

A



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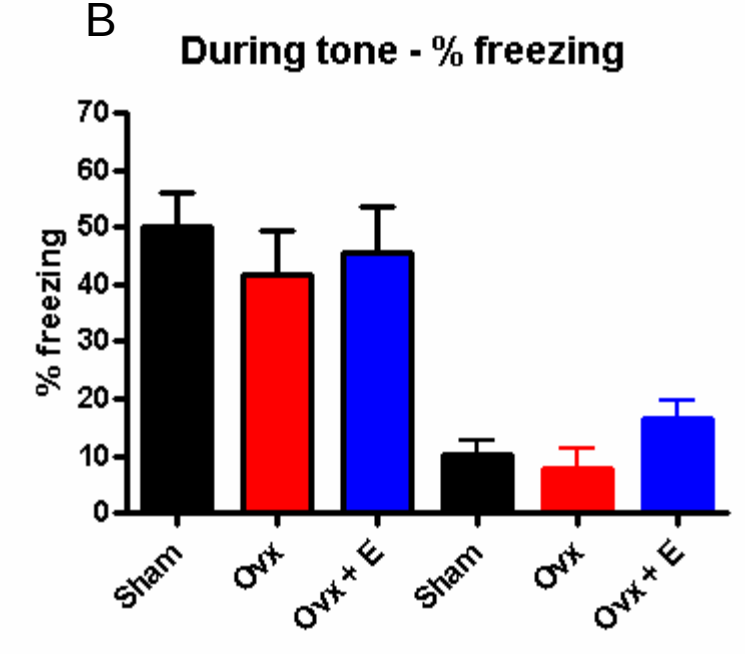

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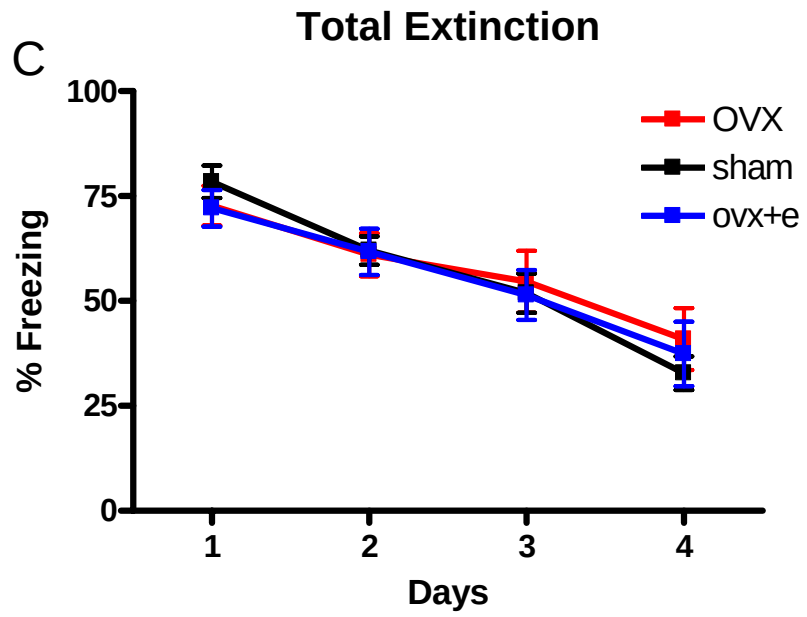


Figure 8 – Hormonal Regulation of Extinction

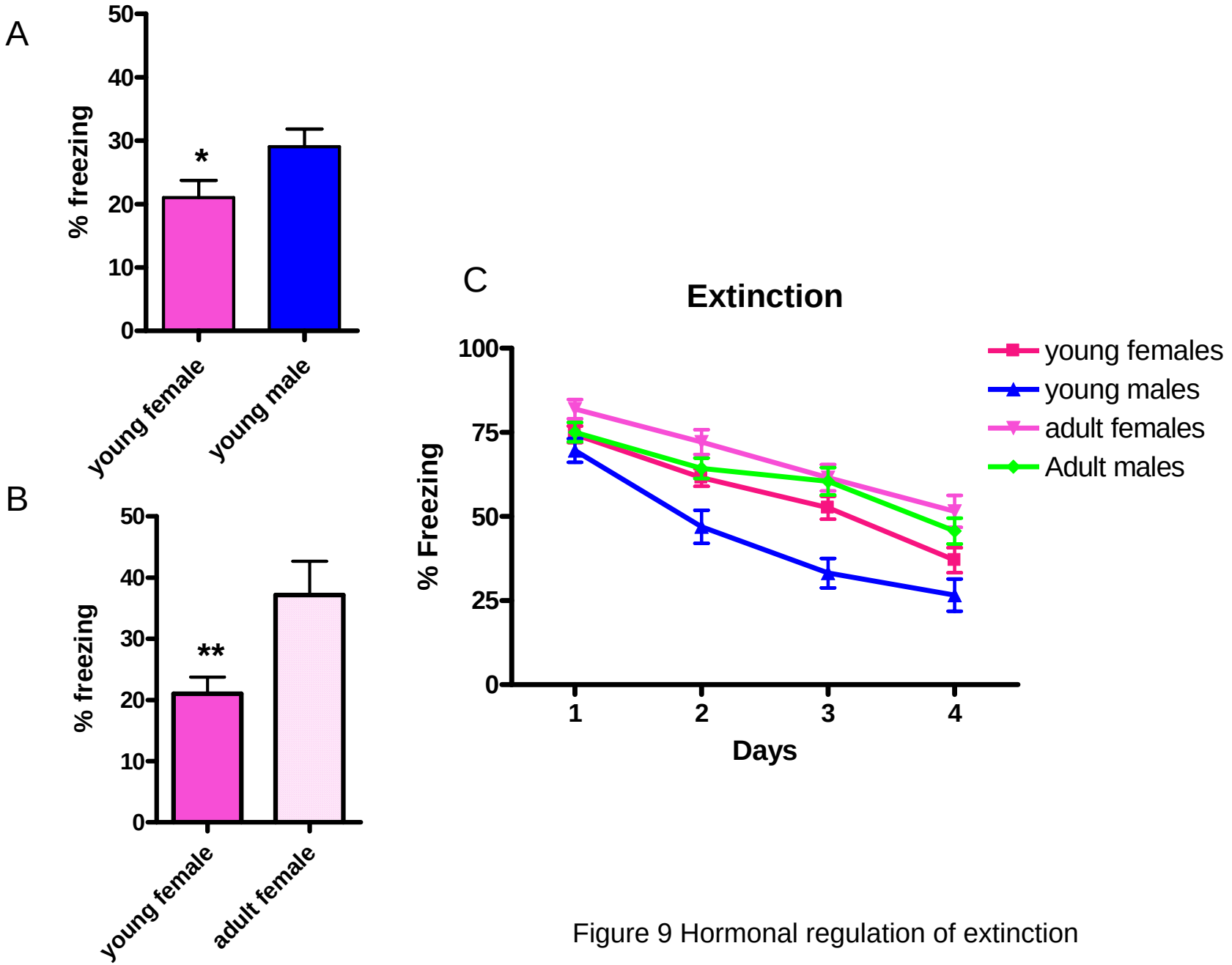
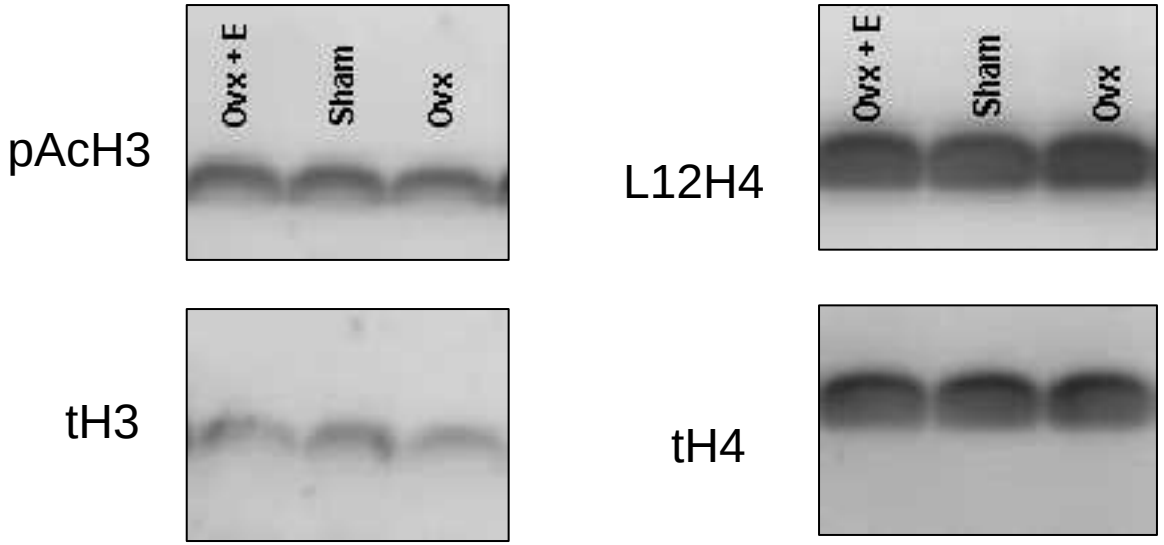
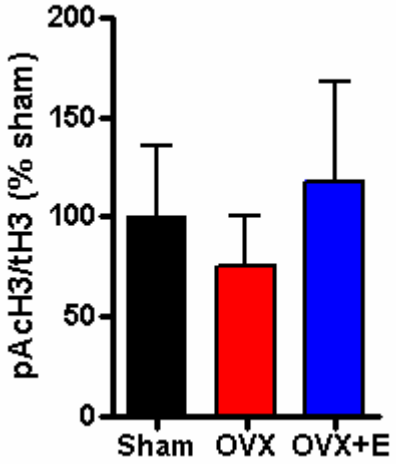


Figure 9 Hormonal regulation of extinction

Adult Females





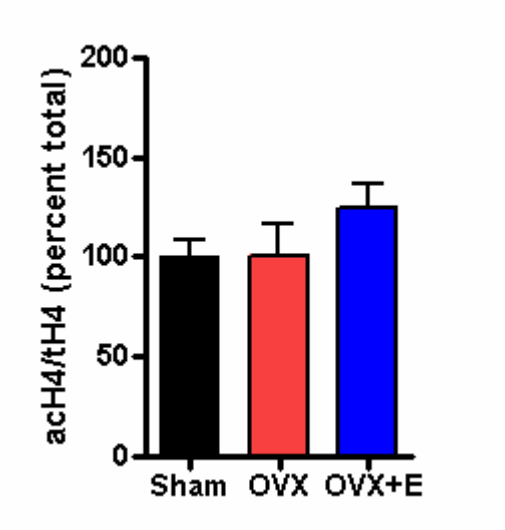
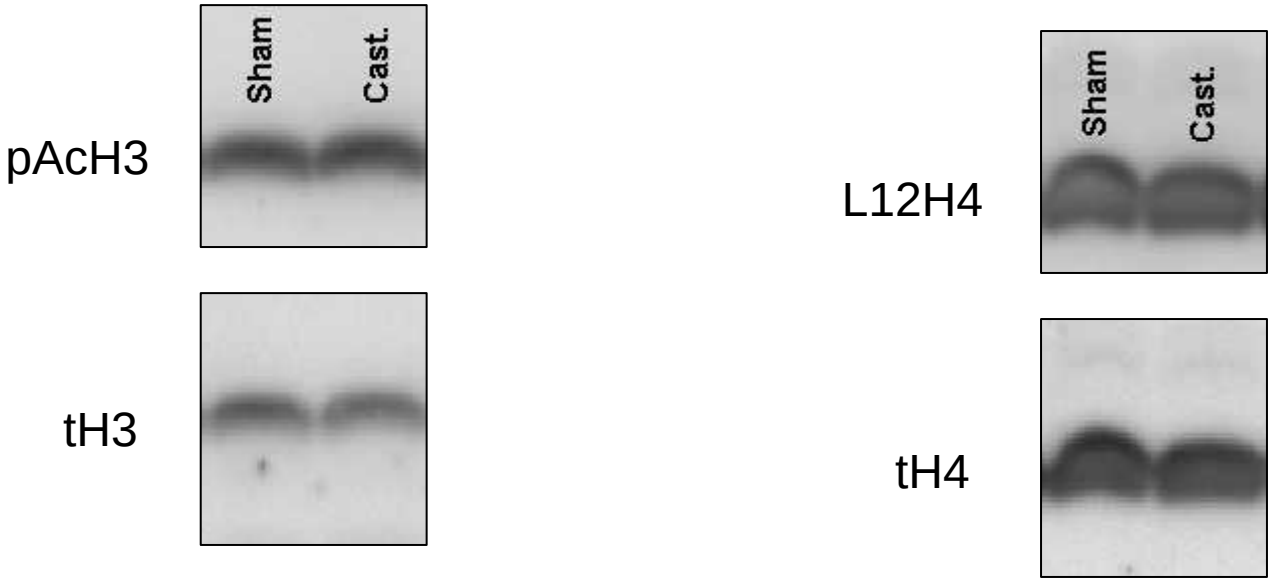


Figure 10: Hormonal regulation of extinction

Adult Males



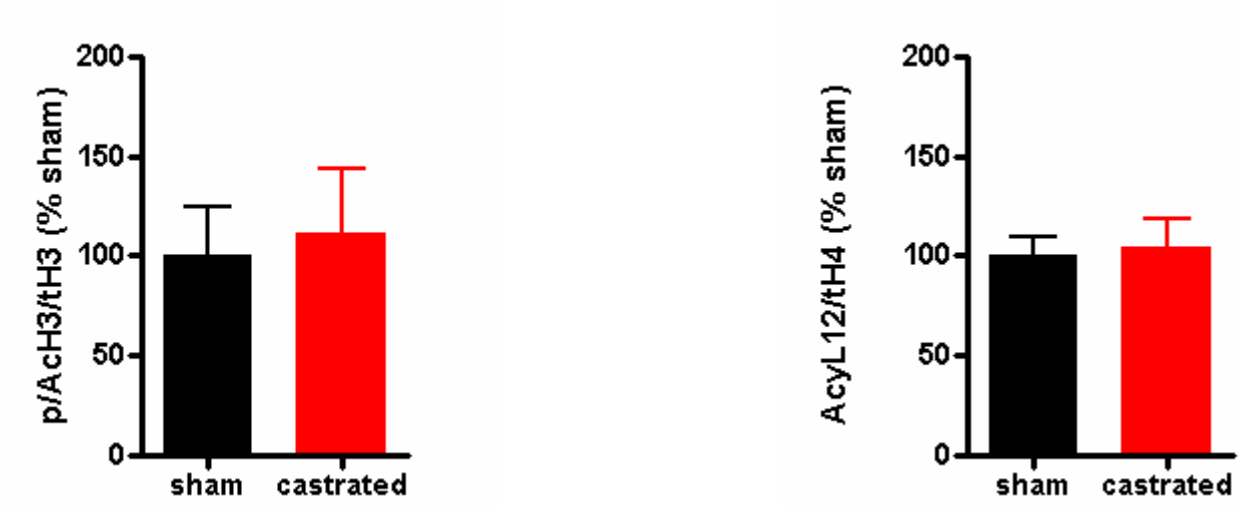


Figure 11: Hormonal regulation of extinction

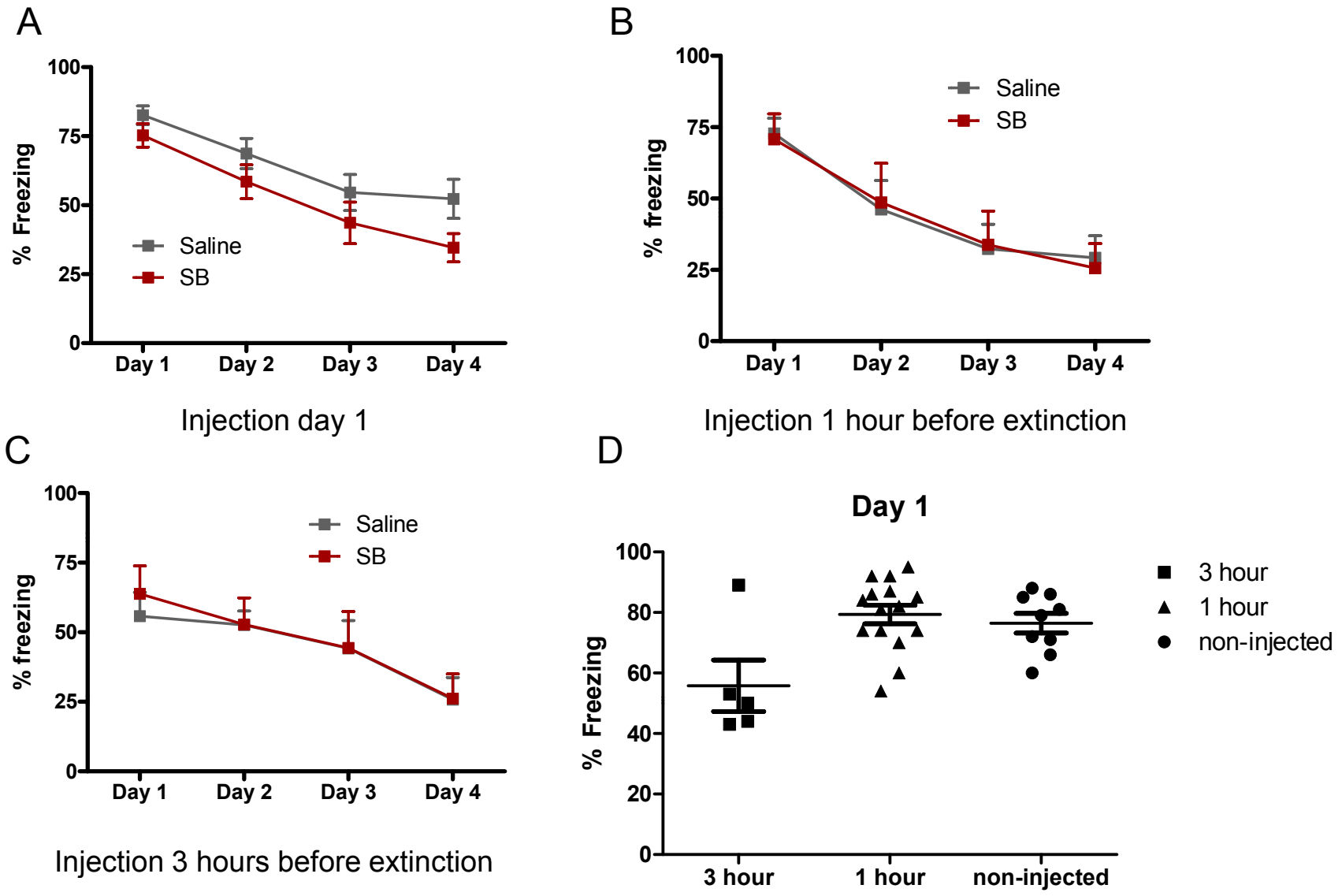


Figure 12. Hormonal regulation of extinction

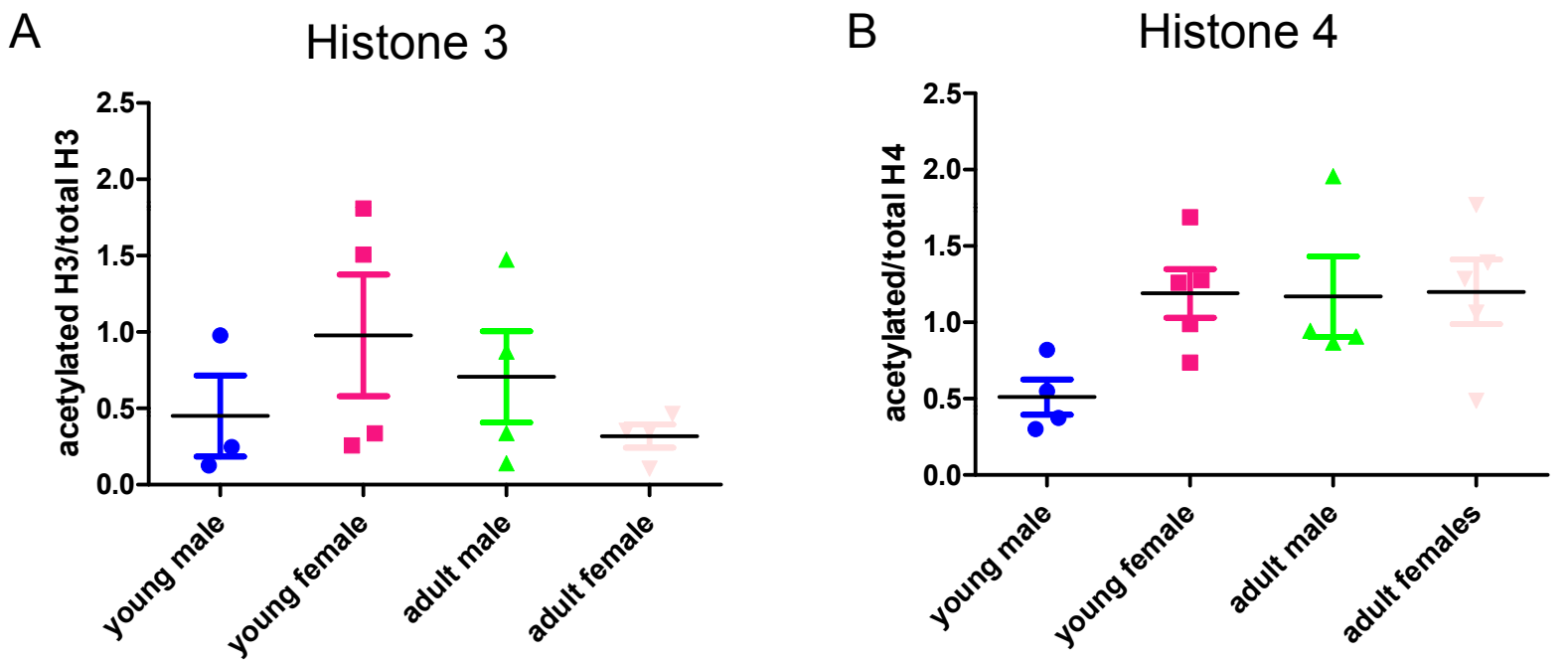


Figure 13. Hormonal regulation of extinction